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Functional characterization of a type 2 metallothionein isoform (OsMTI-2b) from rice

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ABSTRACT

Metallothioneins (MTs) are a family of Cys-rich, low molecular weight, cytoplasmic metal binding proteins. MTs are present in all eukaryotes as well as some prokaryotes. Plant MTs are divided into four types based on Cys distribution pattern in their amino acid sequences. In the present work, the gene encoding OsMTI-2b, a type 2 MT found in rice, was cloned into pET41a vector. The resulting construct was transformed into *Escherichia coli* strain Rosetta (DE3). Following the induction with Isopropyl β-D-1-thiogalactopyranoside the OsMTI-2b was expressed as carboxyl-terminal extensions of glutathione-S-transferase (GST-tag), a 6His-tag, and an S-tag. The expressed recombinant fusion protein was named GST-OsMTI-2b. As compared with control, transgenic *E. coli* cells expressing GST-OsMTI-2b accumulated more Pb^{2+}, Ni^{2+}, Cd^{2+}, Zn^{2+} and Cu^{2+} from culture medium and showed increased tolerance against these metals. Furthermore the *E. coli* cells expressing OsMTI-2b accumulated significantly higher Pb^{2+} than previously made strains which expressing other rice OsMT isoforms. The recombinant GST-OsMTI-2b was purified using affinity chromatography. According to *in vitro* assays the protein GST-OsMTI-2b was able to form complexes with Pb^{2+}, Ni^{2+}, Cd^{2+} and Zn^{2+}. However, the binding ability for the different metals differed in the order: $Pb^{2+} > Cd^{2+} > Zn^{2+} > Ni^{2+}$.

1. Introduction

Metallothioneins (MTs) are intracellular,low molecular weight and Cys-rich proteins present in various eukaryotic organisms, including fungi, plants, invertebrates, mammals and some prokaryotes [1–3]. MTs bind metals through the thiol groups of their Cys residues [4,5].

MTs play important roles in zinc and copper homeostasis and detoxify non-essential trace elements, such as cadmium (Cd) and mercury, because of their characteristic high Cys levels [6]. MTs also protect cells from oxidative stress by intracellular scavenging of free radicals [7]. Because of their high affinities for heavy metals, the roles of MTs in the detoxification of heavy metals and maintaining essential metal ion homeostasis within cells have been widely investigated.

Plant MTs are extremely diverse [8]; and are classified into MT1–MT4 types based on the arrangement of Cys residues [2]. Cys residues in the primary structures of types 1, 2 and 3 MTs are organised into two Cys-rich domains and are separated by a Cys-devoid linker that varies in length depending on the type and source of MT [9]. In these sequences, the variation in the Cys position is largely contained in the N-terminal Cys-rich region, whereas the CXC motif at the C-terminus remains entirely conserved [8,9]. Type 1 MTs generally contain six Cys residues in the N-terminal arranged in a CXC motif, type 2 MTs contain eight Cys residues arranged in CC, CXC and CXXC motifs and type 3 MTs contain four Cys residues with a CXXCXCXDXXC consensus sequence [9,10]. The sequences of the type 4 MTs differ by having a three Cys domain, each containing five or six conserved Cys residues generally arranged as a C-X-C motif [5].

Genes encoding each of the four MT types have been identified in different plant species [11,12]. For example, 11 genes encoding putative MTs have been identified in the rice genome. Five isoforms are grouped based on the phylogenetic relationship between the amino-acid sequences of the putative rice MTs as type 1 (OsMTI-1a, OsMTI-1b, OsMTI-4a, OsMTI-4b and OsMTI-4c), three isoforms as type 2 (OsMTI-2a, OsMTI-2b and OsMTI-2c), two isoforms as type 3 (OsMTII-3a and OsMTI-3b) and one isoform is grouped as type 4 [13]. The genes encoding OsMTs reveal different expression patterns in different tissues, suggesting that each MT isoform has a different function in specific tissues. Our previous studies showed that the heterologous expression of OsMTI-1b [14]; and OsMTI-3a

Abbreviations: DTNB, 5,50-dithiobis (2nitrobenzoic) acid; GST, Glutathione-S-transferase; IPTG, Isopropyl β-D-1-thiogalactopyranose; MT, Metallothionein; Os, *Oryza sativa*; TNB, 2-nitro-5-thiobenzoic acid.

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[15]; confers heavy metal tolerance in *Escherichia coli* and enhances their ability to accumulate heavy metals. However, the heterologous expression of OsMTII-1a, a type 4 MT, does not affect the tolerance of *E. coli* to heavy metals [16].

In this study, we evaluated the metal-binding ability of OsMTI-2b, a type 2 MT, by heterologously expressing the OsMTI-2b isoform in *E. coli* as N-terminal fusion partner of glutathione-S-transferase (GST-tag), a 6His-tag, and an S-tag. The expressed recombinant fusion protein was named GST-OsMTI-2b. The accumulation of metals in *E. coli* expressing GST-OsMTI-2b was evaluated by culturing the cells in a medium containing $CdCl_2$, $ZnSO_4$, $CuSO_4$, $NiCl_2$ or Pb $(CH_3COO)_2$. The purification of considerable quantity of the recombinant OsMTI-2b allowed us to study its binding ability to Ni^{2+}, $Cd^{2+,}$ $Zn^{2+}$and Pb^{2+} *in vitro*.

2. Materials and methods

2.1. Cloning of gene encoding OsMTI-2b

The full-length cDNA of OsMTI-2b (NCBI accession number: AB002820) which was cloned into PME18SFL3 vector was purchased from National Institute of Agrobiological Sciences Data Bank (NIAS Data Bank, Tsukuba, Japan; http://www.dna.affrc.go.jp). The longest open reading frame of 243 bp was amplified from the cDNA using *Pfu* DNA polymerase (Thermo Scientific) in a reaction mixture containing template plasmid, deoxynucleotides, reaction buffer and the primers 5′-ATATGAATTCATGTCGTGCTGCGGTGGC-3′, which carries an *Eco*RI restriction site at the 5′ end (underlined), and 5′-ATATAAGCTTCTAGTTGCAGTTGCAGCAGGAGC-3′ with a *Hin*dIII restriction site (underlined) at the 3′ end. An addition of four bases was included at the 5′ end in each oligonucleotide primer. The thermal profile was as follows: 1 cycle at 95 °C for 5 min; 30 cycles at 94 °C for 1 min; 62 °C for 1 min; 72 °C for 1 min and 1 cycle at 72 °C for 5 min. The PCR product was then digested with enzymes *Eco*RI and *Hin*dIII and ligated into pET-41a as expression vector (Novagen) after linearization with *Eco*RI and *Hin*dIII. The resulting plasmid, termed pET41-OsMTI-3a was verified by sequencing and then introduced in *E. coli* protease-deficient strain Rosetta (DE3) for protein expression. The resulted strain was termed R- MTI-2b. The control strain was also made by transferring plasmid pET41a without gene.

2.2. Heterologous expression and purification

The *E. coli* cells containing either pET41a (Control) or pET41a-OsMTI-2b (R-MTI-2b) were grown at 37 °C in Luria-Bertani (LB) medium in the volume of 50 ml supplemented with 50 μg/ml kanamycin and 5 μg/ml chloramphenicol to an OD_{600} of about 0.6. At this OD cultures were induced by 100 μM Isopropyl β-D-1-thiogalactopyranoside (IPTG). The culture medium was supplemented with 0.6 mM $ZnSO_4{\cdot}7H_2O$ 20 min after addition of IPTG. To confirm heterologous expression of the protein, 1 ml samples of culture medium were harvested by centrifugation 1, 2, 3 and 4 h after addition of IPTG and frozen at −80 °C until use. The frozen pellets were resuspended in 250 μl pre-cold 10 mM Tris–HCl, pH 8.0, disrupted by mild sonication at 4°C and centrifugated at 12,000g, for 20 min. The soluble proteins recovered in supernatant phase were analyzed by 12% SDS-PAGE and stained by Coomasie Brilliant Blue R-250 [17]. For large scale production of proteins the cells were grown in 500 ml of medium. Induction with IPTG, and supplementation with 0.6 mM $ZnSO_4{\cdot}7H_2O$ were performed as explained above. The cells from whole volume were harvested 4 h after addition of IPTG. The soluble proteins were extracted as explained above. For purification, the extracted soluble proteins were applied on to His Trap HP column (GE, Healthcare) preequilibrated with loading buffer (10 mM imidazole, 500 mM NaCl, 30 mM Tris–HCl, pH 8.0) and the bound proteins were eluted by 68.5–283 mM imidazol gradient. Then the imidazole was removed by dialysis. Aliquots of the protein fractions were analyzed by 12% SDS-PAGE and stained by Coomasie Brilliant Blue R-250. The pure fractions were pooled and transferred into 12 kDa molecular weight cutoff cellulose tubes (Sigma) and dialyzed against 10 mM Tris–HCl, pH 8.0, at 4 °C overnight to remove imidazole and other salts. Protein concentrations were determined by amino acid analysis.

2.3. Metal ion tolerance of transformed cells

Tolerance of strains control and R-MTI-2b to metals in the growth medium was examined in concentrations of 0.9 mM $CdCl_2{\cdot}H_2O$, 1 mM $ZnSO_4{\cdot}7H_2O$, 2 mM $CuSO_4{\cdot}5H_2O$, 2.5 mM $NiCl_2$, and 1 mM Pb $(CH_3COO)_2{\cdot}3H_2O$. For the analysis 5 ml of the overnight cultures of cells were inoculated in 80 ml of LB medium supplemented with desired antibiotics. The cultures were induced at OD600 = 0.6 by addition of 0.1 mM of IPTG. After 20 min, the metals were added to the cultures. Bacterial growth was monitored up to 12 h by OD600 measurements. Each data represent the mean obtained from two independent experiments with two replicates.

2.4. Analysis of metal ion accumulation in transformed cells

The growth of cells, induction with IPTG, and supplementation with metals were performed as explained above. For the analysis of metals, cells from 10 ml of culture at 0 (T0) and 6 h after metal addition (T1) were precipitated by centrifugation at 6000 *g* for 20 min. The supernatant was analyzed for metals Zn^{2+}, Cu^{2+}, Ni^{2+}, Cd^{2+} and Pb^{2+} using inductively coupled plasma atomic absorption spectroscopy (PerkinElmer Analyst 700). The metal concentration changes in the medium of strains control and R-MTI-2b between T1 and T0 (C_{T0}-C_{T1}) was calculated. Each data represents the mean ± SD obtained from two independent experiments with two replicates.

2.5. Preparation of apo-protein and reconstitution with different metals

The apo-proteins were prepared and reconstituted with metals previously with slight modifications [18,19]. To this end aliquots of the purified GST-OsMTI-2b protein were acidified with HCl to pH 2.0. The samples were then loaded on Sephadex G-25 column equilibrated by 0.1 N HCl to remove the bound ions. The concentration of protein was then determined after gel filtration. Reconstitution with metals was achieved by the addition of 10 mol equivalents of either Cd^{2+}, Zn^{2+}, Ni^{2+} or Pb^{2+} ions followed by neutralization of the samples to pH 8.0 with 200 mM Tris. The unbound metals were then removed by loading sample on on Sephadex G-25 column equilibrated with10 mM Tris–HCl, pH 8.0.

2.6. UV absorption spectra

The absorption spectra of GST-OsMTI-2b after reconstitution with metals in addition to its apo-form were determined between 220 and 350 nm using spectrophotometer (DU 530).

2.7. Reaction with DTNB

The competitive reaction with 5,50-dithiobis (2nitrobenzoic) acid (DTNB) (Sigma) was carried out by a method previously described [20]. Briefly, 1.5 nmol of each protein which were obtained by reconstitution of apo-form of GST-OsMTI-2b with either Ni^{2+}, Cd^{2+}, Zn^{2+} or Pb^{2+} was diluted in 300 μl of 10 mM Tris–HCl, pH 8.0 and placed into a quartz cuvette. The reaction was